

CLAIMS

We claim:

5 1. A chemiluminescent substrate of a hydrolytic enzyme
having the structure

Lumi-M-P

10 wherein:

- a. Lumi is a chemiluminescent moiety,
- b. M is a multivalent heteroatom, having at least one lone pair electrons, directly attached to said Lumi and to P, and
- c. P is a group that can be removed by hydrolytic enzymes.

2. The chemiluminescent substrate of claim 1 wherein:

20 Lumi is selected from the group consisting of acridinium compounds, benzacridinium compounds, quinolinium compounds, isoquinolinium compounds, phenanthridium compounds, lucigenin compounds, acridans or other reduced forms of the above, acridines or other non-N-alkylated forms of the above, spiroacridan compounds, luminol 25 compounds and isoluminol compounds; and

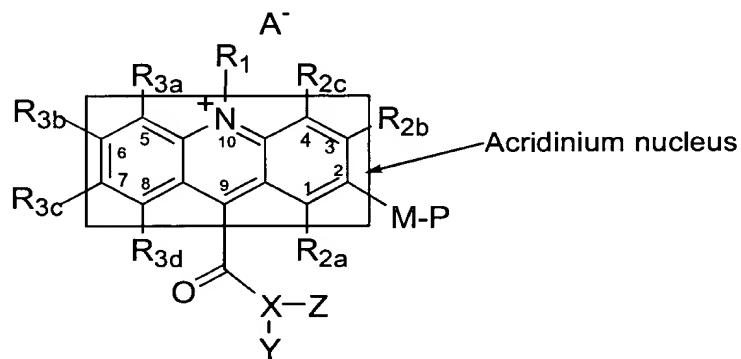
M is selected from the group consisting of oxygen, nitrogen and sulfur.

3. The chemiluminescent substrate of claim 1 wherein

M is a multivalent heteroatom selected from the group consisting of oxygen, nitrogen and sulfur; and

5 P is a group that is thermally and hydrolytically stable in aqueous medium and is removable by a hydrolytic enzyme.

4. The chemiluminescent substrate of claim 2 wherein
said Lumi chemiluminescent moiety is an acridinium
10 compound having the following structure:



wherein:

P is a group that is thermally and hydrolytically
15 stable in aqueous medium and readily removable by a hydrolytic enzyme to form Lumi-M;

M is oxygen, nitrogen or sulfur;

R₁, is an alkyl, alkenyl, alkynyl or aralkyl containing 0 to 20 heteroatoms;

C₁, C₃, C₄, C₅, C₆, C₇ and C₈ peri-positions of the acridinium nucleus are either unsubstituted or substituted, and when substituted, said substitutents R_{2a}, R_{2b}, R_{2c}, R_{3a}, R_{3b}, R_{3c}, and R_{3d} may be the same or different,
5 with said substitutents selected from the group consisting of -R, substituted or unsubstituted aryl, halides, nitro, sulfonate, sulfate, phosphonate, -CO₂H, -C(O)OR, cyano (-CN), -SCN, -OR, -SR, -SSR, -C(O)R, -C(O)NHR, ethylene glycol, and polyethylene glycol, wherein R is selected
10 from the group consisting of alkyl, alkenyl, alkynyl, aryl, and aralkyl having from 0 to 20 heteroatoms;

A⁻ is a counter ion for the electroneutrality of the quaternary nitrogen of the acridinium compounds, said A⁻ not being present if said R₁ substituent contains a
15 strongly ionizable group that can form an anion and pair with the quaternary ammonium cationic moiety;

X is nitrogen, oxygen or sulfur; such that,

when X is oxygen, Z is omitted and Y is a substituted or unsubstituted aryl group or -N=CR₉R₁₀,
20 wherein R₉ and R₁₀ may be the same or different and are selected from hydrogen, substituted or non-substituted aryl, alkyl, alkenyl, alkynyl, halide, alkoxy and aryloxy groups;

when X is sulfur, Z is omitted and Y is a substituted or unsubstituted aryl group;
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when X is nitrogen, Z is -SO₂-Y', Y' being defined the same as Y above; Y is as defined above or can be a branched or straight-chain alkyl containing 0 to 20 carbons, halogenated or

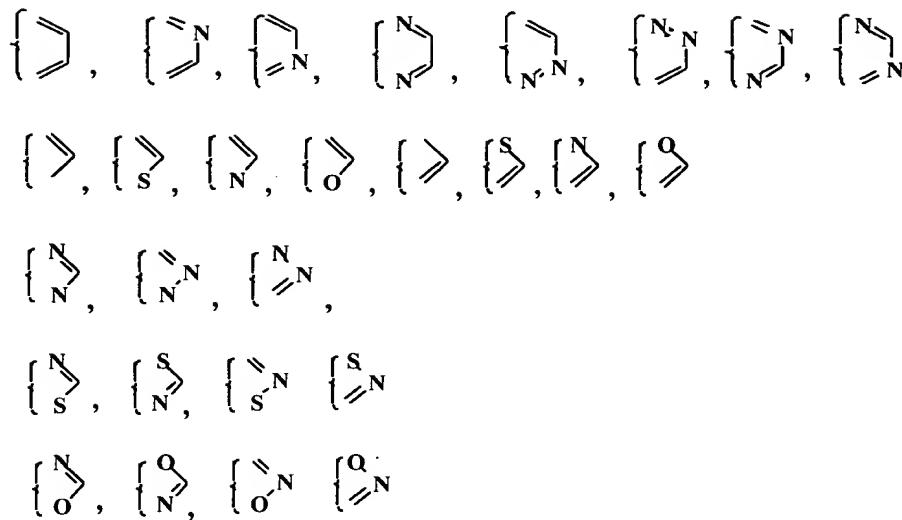
unhalogenated, or a substituted aryl, or heterocyclic ring system; and Y and Y' can be the same or different.

5 5. The chemiluminescent substrate of claim 4 wherein R₁ is methyl, sulfoalkyl or an alkyl containing one or more hydrophilic groups selected from the group consisting of sulfonate, sulfate, -CO₂H, phosphonate, ethylene glycol, polyethylene glycol, quaternary ammonium (-N⁺R₃), and any
10 groups containing one or more of said hydrophilic groups.

6. The chemiluminescent substrate of claim 4 wherein R_{2c}, R_{3a} or R_{3c} is M-P, said C₂ peri-position being substituted or unsubstituted.

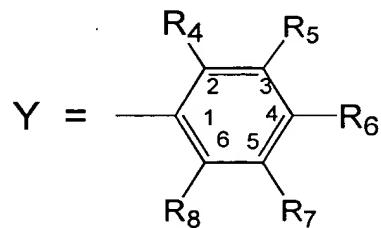
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7. The chemiluminescent substrate of claim 4 wherein any two adjacent substituents at the acridinium nucleus peri-positions can be linked to form additional carbocyclic and heterocyclic rings fused to the attached acridinium nucleus,
20 said rings being selected from the group consisting of:



8. The chemiluminescent substrate of claim 4 wherein said counter ions A are selected from the group consisting of
5 CH_3SO_4^- , FSO_3^- , CF_3SO_3^- , $\text{C}_4\text{F}_9\text{SO}_3^-$, $\text{CH}_3\text{C}_6\text{H}_4\text{SO}_3^-$, halide, CF_3COO^- ,
 CH_3COO^- , and NO_3^- .

9. The chemiluminescent substrate of claim 4 wherein X is oxygen or sulfur, Z is omitted, Y is a polysubstituted aryl
10 group of the following formula:



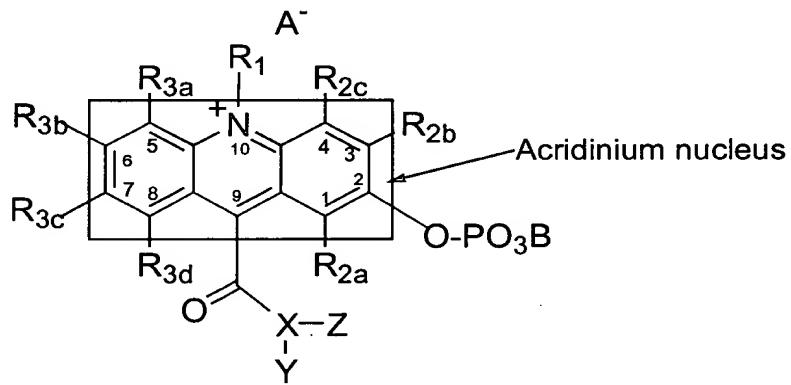
where R_4 and R_8 can be the same or different and are alkyl, alkenyl, alkynyl, alkoxy (-OR), alkylthiol (-SR), or substituted amino groups.

- 5 10. The chemiluminescent substrate of claim 9 wherein R_5 ,
 R_6 and R_7 are the same or different, and are hydrogen, -R,
 substituted or unsubstituted aryl, halides, amino, -
 NHR, -NR₂, quaternary ammonium (-N⁺R₃), hydroxyl, nitro,
 nitroso, sulfonate, sulfate, cyano (-CN), phosphonate,
10 CO₂H, -SCN, -OR, -SR, -SSR, -C(O)R, -C(O)NHR, -NHC(O)R,
 ethylene glycol, or polyethylene glycol.
- 15 11. The chemiluminescent substrate of claim 9 wherein R_4
 and R_8 are short chain alkyl groups containing 1-10 carbons,
 preferably methyl groups, or at least one of R_4 and R_8 is as
 defined while the other is a hydrogen or a halides.
- 20 12. The chemiluminescent substrate of claim 9 wherein any
 adjacent two groups of said R_4 to R_8 can form one or more
 additional fused hydrocarbon aromatic rings or
 heteroaromatic rings with or without substitutions, selected
 from the group consisting of benzene, naphthlene, pyridine,
 thiophene, furan, and pyrrole.
- 25 13. The chemiluminescent substrate of claim 9 wherein R_5 ,
 R_6 and R_7 can be the same or different and comprise
 hydrophilic groups selected from the group consisting of
 sulfonate, sulfate, -CO₂H, phosphonate, ethylene glycol,
 polyethylene glycol, quaternary ammonium (-N⁺R₃), and any
30 groups containing one or more of said hydrophilic groups.

14. The chemiluminescent substrate of claim 4 wherein after the removal of P by a hydrolytic enzyme, M becomes ionizable in the medium of the reaction to bear a negative charge, 5 thus strongly donating electrons to the acridinium ring system.

15. The chemiluminescent substrate of claim 4 having the following structure:

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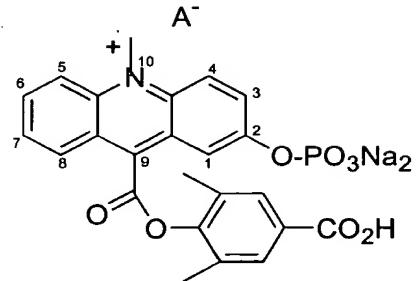


wherein B is either a divalent cation or two monovalent cations, said monovalent cations being the same or different.

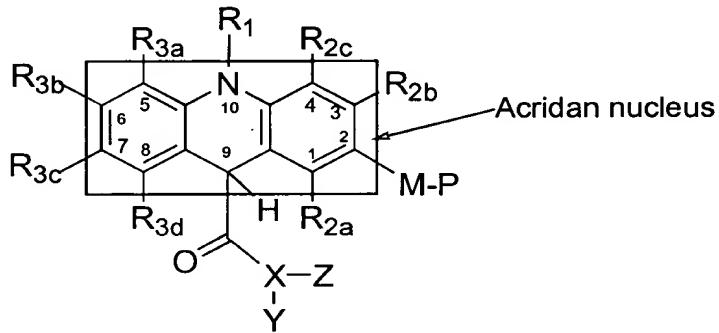
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16. The chemiluminescent substrate of claim 15 wherein, if B is a monovalent cation, each B is selected from the group consisting of sodium, hydrogen, potassium, ammonium, and, or, if B is a divalent cation, B is calcium and 20 magnesium.

17. The chemiluminescent substrate of claim 16 having the following structure:



5 18. The chemiluminescent substrate of claim 2 wherein said chemiluminescent moiety Lumi is an acridan compound having the following structure:

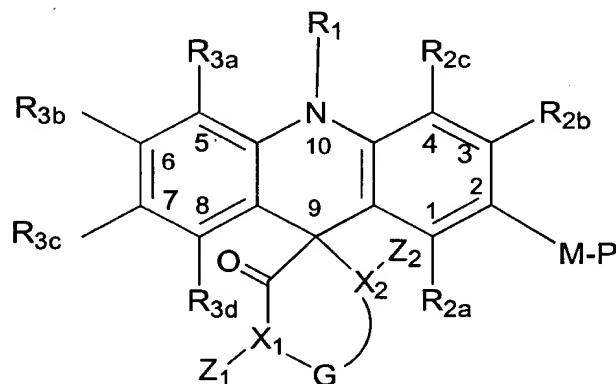


10 wherein, R₁, R_{2a-c}, R_{3a-d}, M, P, X, Y, and Z are as defined in claim 4.

19. The chemiluminescent substrate of claim 18, wherein M-P is O-PO₃Na₂.

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20. The chemiluminescent substrate of claim 2 wherein said chemiluminescent moiety Lumi is a[n] spiroacridan compound, having the following structure:



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wherein, R₁, R_{2a-c}, R_{3a-d}, M and P are as defined in claim 4;

X₁ and X₂ are the same or different and are selected from the group consisting of oxygen, sulfur and nitrogen, 10 and when either one or both of X₁ and X₂ are oxygen or sulfur, the corresponding Z₁ or Z₂ or both Z₁ and Z₂ are omitted; when one or both of X₁ or X₂ are nitrogen, the corresponding Z₁ or Z₂ or both Z₁ and Z₂ are hydrogen, alkyl, aryl or -SO₂-Y'; and

15 G is a group connecting X₁ and X₂ to form a ring having 5 to 10 members.

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A'*